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*THE RELEASE OF AUXIN FROM ISOLATED LEAF PROTEINS
OF SPINACH BY ENZYMES*

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A number of hypotheses have been advanced concerning the substance from which auxin is liberated in plant tissues. Kögl, Erxleben and Haagen Smit⁹ postulated the existence of auxin esters in fats and oils, since they were able to hydrolyze auxin from oil of *Arachis* and other plants by means of lipase or saponification with alcoholic sodium hydroxide. Recently there appeared a brief note by Skoog and Thimann¹³ to the effect that an increase of auxin could be obtained from *Lemna* tissue by the addition of proteolytic enzymes. They considered that the auxin was bound to proteins. Avery, Berger and Shalucha¹ have criticized this hypothesis as being premature since proteolytic enzymes also hydrolyze simple peptides and amides. In addition, they were unable to hydrolyze the auxin precursor in a corn endosperm extract with commercial preparations of trypsin, pepsin, papain, ficin, taka-diastase, malt diastase or steapsin at pH values of 7 or less although the precursor could be hydrolyzed by other means. They suggest that the precursor in maize may be an acid amide of indole acetic acid. It is at once evident that the information concerning this phase of plant hormone research is extremely meager.

Methods for the isolation of proteins from leaves have been proposed by Chibnall,³ Menke¹¹ and Granick,^{5, 6} and it should be possible to test the hypothesis that auxin is associated with protein by the use of isolated plant proteins. If it can be shown that auxin is definitely a part of protein molecules or associated with them, some ramifications of the problem may be studied such as (1) whether auxin is confined to cytoplasmic or chloroplastic protein, or found in both; (2) if it is found in leaf proteins, can these proteins be further fractionated so that the yield of auxin from one fraction is greater than from another; (3) diffusion rates of the auxin released from proteins can be made to determine whether they compare with the values of auxin directly extracted from the leaf. With these considerations in mind, the following experiments were performed.



Preparation of Proteins.—*Cytoplasmic proteins:* Chibnall³ has shown that vacuolar materials, and the soluble substances of the cytoplasm could be removed by cytolysis of the leaf tissue followed by pressure to "squeeze" the cell fluids from the leaf, cytoplasmic protein and chloroplasts remaining within the cell walls. The cells were then broken by grinding, the ground material suspended in water and the cell wall fragments removed by passing the liquid through silk. The chloroplasts were separated from the cytoplasmic proteins by passing the suspension through paper pulp. Menke,¹¹ on the contrary, ground spinach leaves without previous treatment and separated the chloroplastic protein from the cytoplasmic protein by precipitation with 25 per cent ammonium sulphate.

A combination and modification of the methods proposed by Chibnall and Menke have been used to prepare the leaf proteins of spinach for this study. Approximately one kilogram of market spinach (consisting only of leaves) was thoroughly washed and cytolized for a few minutes with ether. The flaccid leaf material was surrounded by cotton cheesecloth and the juice squeezed out in a hydraulic press. Upon releasing the pressure, the leaves were allowed to imbibe water and then pressed again. Imbibition and pressing were repeated three times. After the final pressure treatment, the material was again allowed to imbibe water and the leaves were passed twice through the coarse burr of a "Nixtamal" mill, then three times more using the finest burr. Following this preliminary grinding, water was added in small quantities and the leaf material was repeatedly passed through the mill until a semi-liquid consistency was obtained. The finely divided material was then dispersed in about two liters of distilled water. Cell wall debris was removed by a short centrifugation at low speed and the supernatant liquid was passed through fine mesh silk to remove all visible traces of cell fragments. The combined centrifugates were again ground and dispersed as before and both dispersions combined to give a total of about three liters of liquid.

The chloroplasts were precipitated by adding ammonium sulphate to give a final concentration of 7.5 per cent. The material was allowed to flocculate for 12 hours at 5°C., and the precipitated chloroplasts removed by filtering through Whatman No. 12 folded filter paper. The filtrate was returned through the same paper until the filtrate became free from green coloration. All filtering was done at 5°C. with toluol present to prevent bacterial decomposition of the proteins. We have used 25, 20, 15, 10 and 5 per cent ammonium sulphate to precipitate the chloroplasts in other preparations. Table 1 expresses the nitrogen (and protein) content of some of these preparations. Nitrogen was determined by the micro-Kjeldahl method using selenium as a catalyst. However, smaller yields of cytoplasmic protein than anticipated seemed to indicate that some cytoplasmic protein precipitated with the chloroplasts when high concentrations of

ammonium sulphate were used; hence, the values reported here are confined to those preparations in which not more than 15 per cent ammonium sulphate was used.

TABLE 1

NITROGEN CONTENT OF PROTEINS USED FOR AUXIN ANALYSIS. PROTEINS WERE SUBJECTED TO PROLONGED EXTRACTION WITH ETHER AND ETHER-ALCOHOL BEFORE ANALYSIS (SEE TEXT)

KIND OF PROTEIN	% (NH ₄) ₂ SO ₄ USED TO PRECIPITATE CHLORO- PLASTIC PROTEINS	% N	% PROTEIN (N × 6.25)
Cytoplasmic, precipitated at pH 4.0	15.0	15.6	97.5
	15.0	15.5	96.9
Cytoplasmic, precipitated at pH 4.0	10.0	15.7	98.1
	10.0	15.6	97.5
Cytoplasmic, precipitated at pH 5.2	7.5	15.4	96.3
	7.5	15.5	96.9
Cytoplasmic, precipitated at pH 3.5	7.5	15.5	96.9
Cytoplasmic, precipitated at pH 5.2	5.0	15.2	95.5
	5.0	15.2	95.5
Chloroplastic, prepared by Granick's method	..	11.1	69.4
	..	10.9	68.3

Cytoplasmic protein contained in the filtrate was precipitated by adjusting the pH of the solution to 4.0 with 0.1 *N* hydrochloric acid. All pH measurements were made with a Beckmann glass electrode. The precipitate was allowed to flocculate for several hours and then collected on Whatman No. 1 filter paper. The protein precipitate was washed until free from sulphate with distilled water whose pH had been adjusted to 4.0. The precipitate was then removed from the filter paper and dried in vacuum at 37°C. The dried proteins were ground to pass through a 100-mesh screen and extracted with ether for 72 hours in a Soxhlet apparatus. This extraction was followed by a 1:4 ether-alcohol extraction for 72 hours. Both the ether and ether-alcohol extracts gave negative tests for auxin by the *Avena* assay method. A yield of four grams of cytoplasmic protein has been obtained from one kilogram of leaf tissue when the chloroplasts were precipitated with 7.5 per cent ammonium sulphate.

Chloroplastic proteins: With the object of obtaining a relatively pure sample of chloroplasts, a modification of the differential-centrifugation method suggested by Granick^{5, 6} was used. Seventy-five grams of market spinach were macerated to yield a suspension in glucose solution. Centrifuging seven minutes at 700 RCF threw out the heavy cell debris, crystals, starch granules and clumps of chloroplasts. The supernatant liquid was decanted and centrifuged ten minutes at 1150 RCF to yield a centrifugate with a white layer, probably suspended sand and porcelain, at its lower portion and covered by a layer of chloroplasts. The supernatant liquid of this

deposit was centrifuged fifteen minutes at 1490 RCF to yield a centrifugate of dark green color. Microscopic examination of this showed mostly isolated chloroplasts with an occasional fragment. To remove the glucose, the chloroplasts were suspended and centrifuged twice in distilled water fifteen minutes at 1490 RCF. Then, they were dried *in vacuo* at 37°C., ground to 100 mesh and extracted successively with ether and ether-alcohol. The nitrogen content of this preparation is given in table 1.

Digestions of Proteins by Enzymes.—All digestions of protein herein reported have been carried out under constant conditions. Preliminary experiments indicated that the maximum rate of digestion with tryptic enzymes occurred between a pH of 7.0 and 9.0, using a KH_2PO_4 -NaOH buffer medium. It was likewise found that 72 hours was the minimum time that could be used to digest the proteins and still obtain consistent yields of auxin. Inasmuch as proteolytic enzymes of animal origin appear to attain their maximum efficiency at temperatures near that of blood,⁴ an incubation temperature of 37°C. was used. Digestions were made with 10 mg. of protein suspended in 10 ml. of buffer solution at pH 8.0 with the exception of those digestions employing papain in which 10 ml. of phosphate buffer at pH 4.5 and 6.0 were used. Four to five drops of toluol were added to each digestion flask as an antiseptic and renewed during the digestion period if necessary; each flask was tightly stoppered to prevent evaporation of the toluol. At the completion of the 72-hour digestion period, the pH of the solution was adjusted to 4.0–4.5, the solution transferred to a separatory funnel and shaken twice with 75 to 100-ml. portions of recently distilled ether. Ether containing the auxin was separated from the protein solution and evaporated at low temperature until only a few milliliters remained. These were transferred to a small vial to which agar was added.

Auxin Determinations.—Auxin was determined by the standard *Avena* test procedure,¹⁶ using a reaction time of 90 minutes and a uniform intermediate decapitation time of 2–2.5 hours. All values herein reported are based on determinations made within the dilution range. Dilutions were made either by diluting the agar which contained the hormone, or by dissolving the extract of the hormone in ether, making up to volume with ether at 5°C., and removing suitable aliquots. Although van Overbeek's¹² formula is of utility in *Avena* determinations, the constants involved when 10 to 40-mg. samples are used are of such magnitudes as to make its use impractical in expressing the results obtained here. Hence, the values expressed are actual *Avena* curvatures per 0.35 ml. of agar and comparisons are based upon determinations made on the same day to obviate correcting for differences in sensitivity of the test plants.

Enzymes Used.—We have repeatedly tested various concentrations of the enzymes used for the presence of auxin; the *Avena* assay was always nega-

tive. They were tested before and after 72 hours' incubation under conditions identical with the conditions of protein digestions.

Trypsin and chymotrypsin were commercially prepared crystalline enzymes. Papain† was a twice crystallized preparation dissolved in 0.2M NaCN and had an activity of 10 milk-clotting units per 3.7 mg. of protein. What is called tryptic extract in this paper is a highly purified, non-crystalline trypsin commercially prepared from pancreas and presumably containing a mixture of proteolytic enzymes.¹⁴ Preliminary experiments disclosed no significant difference in auxin yield when 0.5, 1.0 and 5.0 mg. of crystalline enzymes per 10 mg. of protein were used; therefore, 1.0 mg. of crystalline enzyme was used for each digestion with trypsin or chymotrypsin. Similarly, 1.0, 5.0 and 10.0 mg. of tryptic extract were not markedly different in ability to yield auxin. Accordingly, 5.0 mg. of enzyme was added to each digestion in which tryptic extract was used. 1.0 mg. of papain was used in those digestions entailing this enzyme.

Bacterial Contamination.—The possibility exists that auxin liberated from protein may be due to the action of bacteria, either on the protein or on the hydrolytic products of the protein. However, we feel that the following considerations will substantiate our later thesis that the auxin is released by enzymatic hydrolysis and not through the functioning of microorganisms: (1) Toluol was employed as an antiseptic. Any digestion in which the odor of toluol could not be clearly detected at the time of neutralization was discarded. (2) Microscopic examination of the clear liquid from digestions to which toluol had been added disclosed no microorganisms; when toluol was omitted, the liquid became cloudy and its odor offensive; microscopic inspection revealed numerous organisms. (3) No turbidity or odor had developed in a tightly stoppered flask containing 40 mg. of protein, 40 ml. of buffer, 4.0 mg. of chymotrypsin and four drops of toluol at the end of three months. (4) The yields of auxin obtained from like protein and enzyme in various experiments were markedly uniform. That this reproducibility should exist with fortuitous contamination of the digestions by air-borne spores is not probable. (5) Twelve protein digestions were prepared as previously described with trypsin, chymotrypsin and tryptic extract in the presence of toluol. After 72 hours' incubation, 1 ml. of each was plated out upon beef-peptone agar using sterile transfer technique. A few drops of toluol were then added to eight of the plates, none being added to the remaining four. No bacterial or fungus colonies developed within ten days in those plates protected with toluol. Conversely, numerous colonies developed within four days on the four plates not protected by toluol. Undoubtedly the digestions do contain a few microorganisms, but we are convinced that toluol keeps them inactive.

Experimental Results: Hydrolysis of Proteins to Yield Auxin.—Examination of the data presented in table 2 clearly demonstrates that auxin is

associated with protein, and is released through the action of proteolytic enzymes. Like results have also been obtained for three other protein preparations, and always there has been complete corroboration of the data presented. Since the dry protein was suspended in buffer solution, liquefaction of the protein and attainment of a clear solution is assumed to represent hydrolysis. Protein controls, to which no enzyme had been added, failed to dissolve completely during a period of 72 hours, although there sometimes appeared to be a small amount of hydrolysis as attested by *Avena* curvatures. Chibnall has shown that there is a gradual auto-digestion when cytoplasmic proteins are maintained in aqueous solution or in a highly hydrated form. The inference is that intracellular enzymes are present and accomplish this hydrolysis. Thus, one would expect a small amount of auxin to be normally produced in the absence of added enzyme.

TABLE 2

AUXIN RELEASED FROM TWO DIFFERENT PREPARATIONS OF CYTOPLASMIC PROTEINS. DUPLICATES ARE THE RESULT OF TWO SEPARATE *Avena*-CURVATURE DETERMINATIONS WITH TWO SEPARATE PROTEIN DIGESTIONS

PROTEIN PREPARATION	NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE <i>Avena</i> CURVATURE IN DEGREES
Cytoplasmic proteins precipitated at pH 4.0, after precipitation of chloroplastic proteins by 10% (NH ₄) ₂ SO ₄	4*	Control	0
			2.0 ± 0.3
	4	Chymotrypsin	4.4 ± 0.4
			3.8 ± 0.5
	4	Trypsin	6.0 ± 0.4
			4.8 ± 0.6
	4	Tryptic extract	15.0 ± 0.6
			12.5 ± 0.7
Cytoplasmic proteins precipitated at pH 4.0, after precipitation of chloroplastic proteins by 15% (NH ₄) ₂ SO ₄	4	Control	0
			0
	4	Chymotrypsin	2.0 ± 0.9
	4	Trypsin	0
	4	Tryptic extract	8.4 ± 0.8
			9.1 ± 0.4
Cytoplasmic proteins precipitated at pH 5.2, after precipitation of chloroplastic proteins by 7.5% (NH ₄) ₂ SO ₄	3	Control	0
	4	Control	0
			1.3 ± 0.5
	4	Trypsin	0
	2	Chymotrypsin	0
	4	Chymotrypsin	0
	1	Tryptic extract	1.0 ± 0.3
			2.6 ± 0.8
	3	Tryptic extract	2.9 ± 0.6
	4	Tryptic extract	8.5 ± 1.3

* Ether extracts of four separate digestions combined.

When the three enzymes are compared as to their efficacy in causing auxin to be released from protein, tryptic extract (so far as our data go) is the best. Trypsin and chymotrypsin are less effective. Little, if any, visible hydrolysis of proteins resulted when papain was used either at pH 4.0 or 6.0. The results were inconclusive. For instance, one experiment would show an apparently significant increase in auxin but an attempt to duplicate it would fail. The amount of papain available was insufficient to determine the optimum conditions for the hydrolysis of leaf proteins. The most significant fact here, however, is the reproducibility of the results: two separate digestions of equal weight of protein yield nearly equal amounts of auxin as measured by *Avena* curvatures.

Isoelectric Fractionation of Cytoplasmic Proteins.—After the chloroplasts had been removed by precipitation with ammonium sulphate and filtering, 0.1 *N* hydrochloric acid was added to a filtrate containing cytoplasmic proteins until a pH of 5.2 was attained. At this hydrogen-ion concentration, a rapid and voluminous flocculation of protein occurred. Flocculation

TABLE 3

AUXIN YIELD FROM TWO FRACTIONS OF CYTOPLASMIC PROTEINS OBTAINED BY ISO-ELECTRIC PRECIPITATION. (CHLOROPLASTIC PROTEIN HAD BEEN SEPARATED FROM CYTOPLASMIC PROTEIN BY 7.5% $(\text{NH}_4)_2\text{SO}_4$)

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE <i>Avena</i> CURVATURE IN DEGREES PROTEIN PREPARATION	
		PRECIPITATED AT pH 5.2	PRECIPITATED AT pH 3.5
4	Control	1.3 $\pm$ 0.5	5.2 $\pm$ 0.7
1	Control	0	0
1	Chymotrypsin	0	9.8 $\pm$ 1.2
2	Chymotrypsin	0	16.8 $\pm$ 1.0
1	Trypsin	0	6.2 $\pm$ 0.7
		0	1.4 $\pm$ 0.2
1	Tryptic extract	1.0 $\pm$ 0.3	15.3 $\pm$ 1.1
		1.3 $\pm$ 0.4	

was allowed to proceed at room temperature for one-half hour, and the precipitate was then removed by filtration at 5°C. which required about six hours. The filtrate was then adjusted to pH 3.5 which precipitated more protein. This was removed by filtration. Both precipitates were thoroughly washed with water properly acidified, dried *in vacuo* at 37°C. and extracted with ether and ether-alcohol. The protein fraction precipitated at pH 3.5 constituted about five per cent of the total cytoplasmic protein. Auxin yields obtained by enzymatic hydrolysis of these two cytoplasmic protein fractions appear in table 3.

When considered on a basis of protein weight, it will be noticed that the protein fraction precipitated at pH 3.5 contains considerably more auxin than the protein precipitated at pH 5.2. Here again, the same trend is evident: tryptic extract releases more auxin than trypsin or chymotrypsin.

The presence of intracellular enzymes capable of hydrolyzing proteins and releasing auxin is suggested by the relatively large curvature obtained in the control without enzymes using four 10 mg. digestions of protein. However, the control consisting of one 10 mg. digestion of protein should be used for comparison with those digestions to which enzymes were added.

Auxin Yield from Chloroplastic Proteins.—The values in table 4 disclose that auxin is also obtained by enzymatic hydrolysis from a protein preparation of spinach chloroplasts. From table 1, it is obvious that the chloroplastic protein preparation is of lower purity than the cytoplasmic protein preparations. If the curvatures in table 4 are weighted on a per-milligram-of-nitrogen basis, a better comparison can be obtained between the auxin content of the two proteins. Of course, this is made on the assumption that auxin is associated only with the protein of the chloroplasts and cytoplasm. The values for auxin obtained from chloroplastic protein were increased by 40 per cent; table 5 shows these curvatures weighted to allow for impurities. Compensating for the impurity in the chloroplastic protein, the two proteins yielded approximately the same amount of auxin with trypsin and tryptic extract, but not with chymotrypsin.

TABLE 4
AUXIN YIELD FROM CHLOROPLASTIC AND CYTOPLASMIC PROTEINS

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE <i>Avena</i> CURVATURE IN DEGREES	
		CHLOROPLASTIC PROTEIN (BY GRANICK'S METHOD)	CYTOPLASMIC PROTEIN PRECIPITATED AT pH 3.5
2	Control	2.6 $\pm$ 0.6	3.5 $\pm$ 0.4
1	Control	0	0
1	Trypsin	3.9 $\pm$ 0.5	6.2 $\pm$ 0.7
2	Chymotrypsin	4.5 $\pm$ 0.9	16.8 $\pm$ 1.0
1	Chymotrypsin	0.5 $\pm$ 0.2	9.8 $\pm$ 1.2
1	Tryptic extract	15.8 $\pm$ 1.0	15.3 $\pm$ 1.1
		11.6 $\pm$ 0.8	

TABLE 5
AUXIN YIELD FROM CHLOROPLASTIC AND CYTOPLASMIC PROTEINS COMPARED ON A PER-MILLIGRAM-OF-NITROGEN BASIS

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE <i>Avena</i> CURVATURE IN DEGREES	
		CHLOROPLASTIC PROTEIN	CYTOPLASMIC PROTEIN
2	Control	3.6	3.5
1	Control	0	0
1	Trypsin	5.5	6.2
2	Chymotrypsin	6.3	16.8
1	Chymotrypsin	0.7	9.8
1	Tryptic extract	19.2*	15.3

* Average of two determinations.

Diffusion Experiments.—The question can be raised whether the auxin released from the proteins is similar to the auxin found in the leaf. Corresponding diffusion rates of the two auxins should indicate a similarity in

molecular weights. The diffusion technique has been described by a number of workers;^{8, 15} accordingly, experiments were performed in general following previous procedures comparing the auxin obtained from ether extracts of spinach leaves to the auxin liberated by enzymatic hydrolysis of the cytoplasmic protein. At the same time, these auxins were compared with a growth substance of known constitution, crystalline indole acetic acid. Agar blocks containing the auxin preparations, purified by

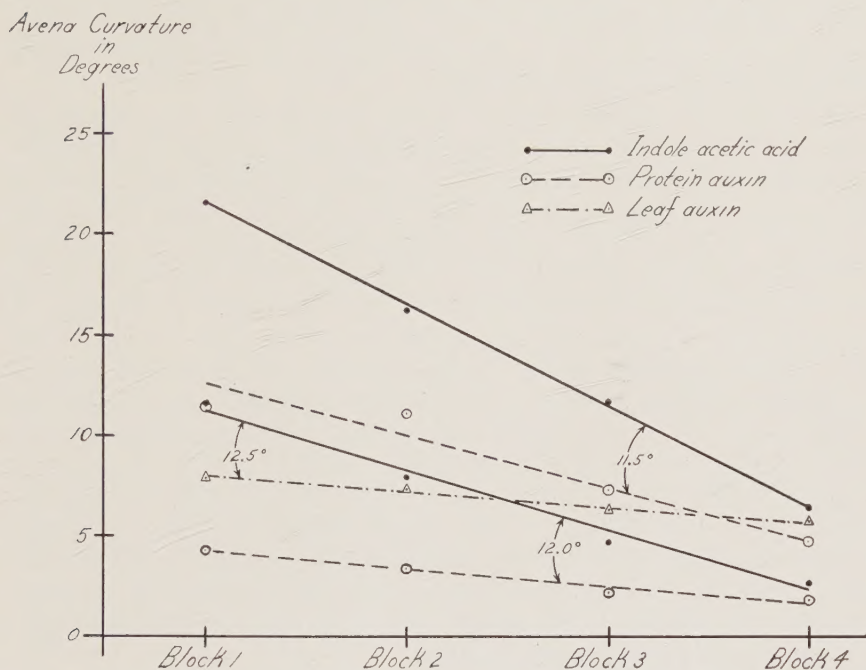


FIGURE 1

The distribution of auxin in four agar blocks, each 1.6 mm. thick, after 90 minutes' diffusion. The two upper curves represent one experiment, while the three lower curves are from another experiment. The initial concentration of indole acetic acid was 100 gammas per liter.

the donor-block method suggested by van Overbeek,¹² were placed upon three blocks of pure agar, each 1.6 mm. thick, and diffusion was allowed to ensue for 90 minutes.

If the concentrations of auxin in the four blocks are within the proportionality range, the resulting *Avena* curvatures plot graphically as a straight line. The slope of this line is dependent upon the time of diffusion, the temperature and the weight of the auxin molecule. If the temperature and time of diffusion are held constant for the auxins under comparison in a

given experiment, the slopes should vary as a function of the molecular weights. Hence, we have used contrasting slopes as a criterion of dissimilar molecular weights.

In figure 1 are shown data from two diffusion experiments. The angular difference between the indole acetic acid and enzyme auxin curves of one experiment is 11.5° ; in the other experiment, it is 12.0° , and the angular difference between the leaf auxin and indole acetic acid curves is 12.5° . Parallel data have been obtained from repeated experiments. There is a dissimilarity between the slopes of the curves obtained with indole acetic acid and enzyme auxin, and a similarity between the slopes of the curves obtained with enzyme and leaf auxins.

Discussion and Interpretation of Experimental Results.—The preceding data establish that an *Avena* growth-accelerating substance is definitely associated with proteins isolated from the leaves of spinach; the auxin can be released from the proteins through the action of various proteolytic enzymes. Tryptic extract appears to yield a greater amount of auxin than crystalline trypsin or chymotrypsin. This result would indicate that the absolute amount of auxin released depends upon the extent of hydrolysis of the proteins, if it is assumed that tryptic extract contains a mixture of at least three proteolytic enzymes, trypsin, chymotrypsin and carboxypolypeptidase² and hence, will split a protein more completely than the crystalline enzymes. However, it is not argued that the enzymes used in this study represent those present in the plant. On the contrary, we consider it more plausible that the plant contains other enzymes which function to release auxin from proteins. These results explain at least in part the hydrolytic release of auxin from plant material reported by Gustafson,⁷ Link, Eggers and Moulton¹⁰ and others. The elucidation of the position that auxin occupies in the protein or in association with the proteins must, of course, await further experimentation.

The possibility that association of auxin with proteins is an adsorption phenomenon should be considered. We feel that the evidence weighs against a hypothesis of purely physical adsorption. In the first place, auxin released from proteins by hydrolysis is organophilic; hence, if it were adsorbed on proteins, prolonged extraction with organic solvents should elute the auxin from the protein. No auxin has been obtained by extraction of the proteins with ether and ether-alcohol. Second, liquefaction of proteins by enzymes is complete within twelve hours, but auxin cannot be obtained consistently until the proteins have been hydrolyzed for 72 hours. If the process is one of adsorption, it might reasonably be assumed that auxin would be released as soon as the protein had been liquefied.

It seems unlikely that substances of low molecular weight such as amides, peptides, etc., should be contained in these protein preparations. The washings after cytotoxicity of the leaf to remove cell fluids, together with

the narrow pH range of cytoplasmic protein precipitation, and extensive washing of the precipitates throughout the process of preparation would tend to remove water soluble impurities.

We hold that our experiments are valid for spinach proteins and are verifiable on the same material. Although one might extend these results to other leaf material on the basis of Chibnall's work which showed that the differences were "strikingly small" between the leaf proteins prepared from five separate plant families, one would certainly not be justified without extensive further work in drawing an analogy between these proteins and those contained in seeds, fruits, etc.

The consideration that auxin is found in both the chloroplastic and cytoplasmic proteins leads to an interesting speculation: does auxin function in the synthesis of these proteins, or is auxin represented as an end-product of protein metabolism? If the former hypothesis can be substantiated, then the presence of auxin in both fractions can perhaps be explained. If the latter is found to be the true explanation, why is auxin not confined to the cytoplasm which is in contact with the cell wall upon which its function has been explained, according to Went and Thimann, by Heyn?

It has been shown that the auxin content of one fraction of cytoplasmic protein is greater than in another when the two fractions have been separated from each other by isoelectric precipitation. This fact at once suggests the desirability of separating and crystallizing these proteins. Not until this has been accomplished can an adequate study of the amount of auxin released in relation to the extent of protein hydrolysis be undertaken, since at the present stage it is impossible to tell if auxin is associated with some proteins, but not with others, or associated with all proteins but in varying amounts. Chibnall believes that protein preparations of leaves "are really mixtures of many proteins which have similar solubility properties" (p. 141), although they can be conveniently classified as glutelins on the basis of their insolubility in water and solubility in a slight excess of either acid or base. It may also be possible to isolate the enzymes responsible for auxin release in the plant since they are apparently present in some of the protein preparations.

The identification of the auxin released by enzymatic hydrolysis of the leaf protein with the free auxin of the leaf can only be determined by actual isolation of the auxins. From the data presented in figure 1, and other experiments, it appears that the auxin released from proteins is of similar molecular size to the free auxin of the leaf. The dissimilarity between the slopes of the curves for enzyme and leaf auxin, on the one hand, as contrasted with those for indole acetic acid on the other suggests that the auxin in the leaf and the auxin released from proteins are of a lower molecular weight than indole acetic acid.

Summary.—1. Auxin has been shown to be associated with proteins isolated from the leaves of spinach. It can be released by enzymatic hydrolysis.

2. Auxin is obtained from both the cytoplasmic and chloroplastic proteins by enzymatic hydrolysis of the proteins. The cytoplasmic proteins have been separated into two fractions by isoelectric precipitation; more auxin is obtained from one of these fractions than from the other.

3. Diffusion experiments indicate the similarity between the leaf auxin and the auxin released from proteins by enzymatic hydrolysis. Both auxins appear to be of lower molecular weight than indole acetic acid.

4. Evidence is presented that the auxin released from leaf proteins of spinach is not a result of bacterial contamination.

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